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Protection of White Leghorn chickens by U.S. emergency H5 vaccination against clade 2.3.4.4 H5N2 high pathogenicity avian influenza virus

Kateri Bertran, Charles Balzli, Dong-Hun Lee, David L. Suarez, Darrell R. Kapczynski, David E. Swayne *

Exotic and Emerging Avian Viral Diseases Research Unit, Southeast Poultry Research Laboratory, US National Poultry Research Center, Agricultural Research Service, US Department of Agriculture, 934, College Station Rd, Athens, GA 30605, United States

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ABSTRACT

During December 2014–June 2015, the U.S. experienced a high pathogenicity avian influenza (HPAI) outbreak caused by clade 2.3.4.4 H5Nx Goose/Guangdong lineage viruses with devastating consequences for the poultry industry. Three vaccines, developed based on updating existing registered vaccines or currently licensed technologies, were evaluated for possible use: an inactivated reverse genetics H5N1 vaccine (rgH5N1) and an RNA particle vaccine (RP-H5), both containing the hemagglutinin gene of clade 2.3.4.4 strain, and a recombinant herpesvirus turkey vectored vaccine (rHVT-H5) containing the hemagglutinin gene of clade 2.2 strain. The efficacy of the three vaccines, alone or in combination, was assessed in White Leghorn chickens against clade 2.3.4.4 H5N2 HPAI virus challenge. In Study 1, single (rHVT-H5) and prime-boost (rHVT-H5 + rgH5N1 or rHVT-H5 + RP-H5) vaccination strategies protected chickens with high levels of protective immunity and significantly reduced virus shedding. In Study 2, single vaccination with either rgH5N1 or RP-H5 vaccines provided clinical protection in adult chickens and significantly reduced virus shedding. In Study 3, double rgH5N1 vaccination protected adult chickens from clinical signs and mortality when challenged 20 weeks post-boost, with high levels of long-lasting protective immunity and significantly reduced virus shedding. These studies support the use of genetically related vaccines, possibly in combination with a broad protective priming vaccine, for emergency vaccination programs against clade 2.3.4.4 H5Nx HPAI virus in young and adult layer chickens.

1. Introduction

In the past decades, high pathogenicity avian influenza (HPAI) has become one of the major zoonotic health issues that commercial poultry, wildlife, and humans have faced worldwide [1–3]. Outbreaks of HPAI in poultry and wild birds across continents have had a dramatic economic and social impact [4,5]. During December 2014–June 2015, the U.S. experienced the worst HPAI event for its poultry industry, with 21 states reporting Eurasian A/goose/Guangdong/1/1996 (Gs/GD) lineage HPAI H5N8, and Eurasian/North American reassortant H5N2 and H5N1 subtypes of clade 2.3.4.4 in commercial premises, backyard flocks, and wild birds [6–8]. Almost 50 million birds, primarily meat turkeys and layer chickens, died or were culled during the eradication program [9] and exports of U.S. poultry and poultry products to many different countries were banned [10], renewing interest in the development of vaccines for emergency use as preventative measure.

To reduce the economic impact on agriculture and a potential pandemic for humans, development of safe and effective vaccines that can protect from HPAI clinical disease and reduce or eliminate viral shedding in exposed birds has been recognized as a viable alternative approach to culling by decreasing risk of transmission in poultry and, ultimately, to humans [11,12]. The use of vaccines is a justifiable tool for control of HPAI when implemented properly

Abbreviations: APHIS, Animal and Plant Health Inspection Service; ABSL-2, animal biosafety level 2; ABSL-3E, animal biosafety level 3 enhanced; Gs/GD, Asian-origin A/goose/Guangdong/1/1996; Tk/MN/15, A/turkey/Minnesota/12582/2015 (H5N2); AI, avian influenza; CVB, Center for Veterinary Biologics; dpc, days post-challenge; DIVA, differentiation of infected from vaccinated animals; GMT, geometric mean titers; HI, hemagglutinin inhibition; HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza; MDT, mean death time; EID_{50}, mean embryo infectious doses; NVS, National Veterinary Stockpile; RRT-PCR, quantitative real-time RT-PCR; rFPV-H5, recombinant fowl-pox virus vectored vaccine; rHVT-H5, recombinant herpesvirus turkey vectored vaccine; rgH5N1, reverse genetics H5N1 vaccine; RP-H5, RNA particle vaccine; SEPRL, Southeast Poultry Research Laboratory; SPF, specific pathogen free; WS/05, A/Whooper Swan/Mongolia/3/2005 (H5N1).

* Corresponding author.

E-mail addresses: Kateri.Bertran@ars.usda.gov (K. Bertran), Charles.Balzli@ars.usda.gov (C. Balzli), Donghun.Lee@ars.usda.gov (D.-H. Lee), David.Suarez@ars.usda.gov (D.L. Suarez), Darrell.Kapczynski@ars.usda.gov (D.R. Kapczynski), David.Swayne@ars.usda.gov (D.E. Swayne).

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and in combination with strict epidemiological surveillance and biosecurity measures [5]. Indeed, immunization has been a more cost-effective and feasible tool than stamping-out programs alone in some developing countries [13–15]. Vaccination was not implemented in the 2014–2015 U.S. outbreak; however, the outline of an emergency vaccine bank and vaccination policy for use in the future may be strategic to shorten the time for development and approval for vaccines. Vaccines for inclusion in the U.S. National Veterinary Stockpile (NVS) and their potential field use should only be considered under certain conditions: (1) if the vaccine matching the circulating strains is available and targeted to high-risk poultry populations; and (2) if the vaccine enables or has the potential for differentiation of infected from vaccinated animals (DIVA) [12,16].

The objective of this study was to assess the immunogenicity and protective efficacy of three NVS-registered H5Nx vaccines, alone or in combination: an inactivated reverse genetics H5N1 vaccine (hereafter rgH5N1) and an RNA particle vaccine (hereafter RP-H5), both containing the hemagglutinin (HA) gene of A/Gyrfalcon/Washington/40188-6/2014 (H5N8) clade 2.3.4.4 strain, and a recombinant herpesvirus turkey vectored vaccine (hereafter rHVT-H5) containing the HA gene of A/Swan/Hungary/4999/2006 (H5N1) clade 2.2 strain. Studies were performed in day-old chicks and adult White Leghorn (egg laying-type) chickens against lethal clade 2.3.4.4 HPAI virus challenge.

2. Materials and methods

2.1. Animals

Eighty specific pathogen free (SPF) day-old chicks (for Study 1) and 110 SPF adult 61-week-old White Leghorn hens (for Studies 2 and 3) from the Southeast Poultry Research Laboratory (SEPRL) flock were utilized. Each group of birds was housed separately in negative pressured isolators with HEPA-filtered air within the animal biosafety level 2 (ABSL-2) facilities of SEPRL during the vaccination period. Subsequently, they were transferred to animal biosafety level 3 enhanced (ABSL-3E) facilities, housed in negative pressure HEPA-filtered isolators for the challenge period as indicated in Table 1. Birds had *ad libitum* access to feed and water throughout the experiment. All procedures were performed according to the requirements of the protocol approved by the Institutional Laboratory Animal Care and Use Committee.

2.2. Vaccines

Three types of vaccines were tested. First, an inactivated rgH5N1 vaccine contained the HA gene from A/Gyrfalcon/Washington/40188-6/2014 (H5N8) clade 2.3.4.4 strain with the polybasic cleavage site of the HA gene altered to a typical cleavage site sequence of low pathogenicity avian influenza (LPAI) virus, and the remaining 7 backbone segments obtained from the A/Puerto Rico/8/1934 (H1N1) common vaccine strain. The rgH5N1 virus was inactivated with 0.1% β-propiolactone (Sigma Aldrich, St. Louis, MO) and used to prepare an oil-in-water vaccine utilizing a mineral oil-based emulsion (Montanide ISA 70VG, SEPPIC, Paris, France) as previously described [17–20]. The vaccine was administered subcutaneously in a dose of 512 HA units/0.5 ml per bird. Second, a rHVT-H5 vaccine (Vectormune® AI, Ceva Animal Health, Lenexa, KS) was constructed by inserting the HA gene of the HPAI virus A/Swan/Hungary/4999/2006 (H5N1) clade 2.2 strain, with a modified cleavage site compatible with LPAI, into the genome of HVT FC-126 strain. The vaccine was prepared and administered subcutaneously in a dose of 2000 pfu/0.2 ml per bird, as per manufacturer's instructions. Finally, the RP-H5 vaccine (AlphaVax, Merck Animal Health, Ames, IA) contained the HA gene from A/Gyrfalcon/Washington/40188-6/2014 (H5N8) with a modified cleavage site compatible with LPAI. The vaccine was prepared and administered intramuscularly in a dose of $10^{7.0}$ RNA particles/0.5 ml per bird, as per manufacturer's instructions.

2.3. Virus

The influenza A isolate A/turkey/Minnesota/12582/2015 (H5N2) (Tk/MN/15) was used as challenge virus. The Tk/MN/15 virus was selected because it is poultry-adapted and is representative of the Midwest H5N2 cluster both phenotypically [21] and phylogenetically [22]. The virus was propagated and titrated by

Table 1
Summary of studies.

Study	Age	Vaccines[1] (age[2])	No. birds	Age[2] at challenge	Survivability[3]	Peak oral shedding (2 dpc)[4]	HI serology (pre-challenge)[5]: Vaccine strain as antigen	HI serology (pre-challenge)[5]: Challenge strain Tk/MN/15 as antigen
Study 1	Day-old	Sham	10	4w	0/10^a	10/10 ($10^{6.0}$)^a	0/10 (<2^3)	nd
		rHVT-H5 (1d)	10	4w	9/10^b (90%)	4/10 ($10^{1.9}$)^b	10/10 ($2^{4.5}$)	10/10 ($2^{3.3}$)
		Sham	20	7w	0/20^a	20/20 ($10^{6.0}$)^a	0/20 (<2^3)	nd
		rHVT-H5 (1d) + rgH5N1 (4w)	20	7w	20/20^b (100%)	3/20 ($10^{1.9}$)^b	20/20 ($2^{8.6}$ rgH5N1; $2^{9.6}$ WS/05)	20/20 ($2^{8.0}$)
		rHVT-H5 (1d) + RP-H5 (4w)	20	7w	20/20^b (100%)	5/20 ($10^{1.9}$)^b	20/20 ($2^{8.3}$ rgH5N1; $2^{8.0}$ WS/05)	18/20 ($2^{4.7}$)
Study 2	Adult	Sham	30	64w	0/30^a	30/30 ($10^{6.4}$)^a	0/30 (<2^3)	nd
		rgH5N1 (61w)	20	64w	20/20^b (100%)	9/20 ($10^{1.9}$)^b	20/20 ($2^{5.9}$)	20/20 ($2^{6.5}$)
		RP-H5 (61w)	20	64w	19/20^b (95%)	16/20 ($10^{3.1}$)^c	15/20 ($2^{5.6}$)	nd
Study 3	Adult	Sham	20	84w	0/14^a	14/14 ($10^{7.7}$)^a	0/14 (<2^3)	nd
		rgH5N1 (61w) + rgH5N1 (64w)	20	84w	17/17^b (100%)	7/17 ($10^{2.7}$)^b	17/17 ($2^{9.4}$)	nd

[1] rgH5N1 = inactivated oil emulsion vaccine with reverse genetic H5 gene insert from clade 2.3.4.4 (512 HAU/dose); rHVT-H5 = live recombinant herpesvirus turkey vaccine with H5 gene insert from clade 2.2 (512 HAU/dose); RP-H5 = RNA particle vaccine with H5 clade 2.3.4.4 hemagglutinin ($10^{7.0}$ RNA particles/dose); Tk/MN/15 = A/turkey/Minnesota/12582/2015 (H5N2); WS/05 = A/Whooper Swan/Mongolia/3/2005 (H5N1).

[2] d = day old; w = weeks old.

[3] Different superscript lowercase denotes statistically significant differences between vaccine and corresponding sham ($p < 0.05$); the numbers represent no. survivors/total.

[4] The numbers represent no. virus positive/total in group followed by mean virus shed titer. Different superscript lowercase denotes statistical significance of number of birds shedding between vaccine and corresponding sham by Fisher Exact or Chi square tests ($p < 0.05$). Different superscript uppercase denotes statistical significance of shedding titers between vaccine and corresponding sham by Mann-Whitney test ($p < 0.05$).

[5] The numbers represent no. serology positive/total in group followed by mean HI titers against vaccine virus or challenge virus. Positive defined as titers $\geq 2^3$. nd = not determined.

allantoic sac inoculation of 9 day-old embryonating chicken eggs by standard methods [23].

2.4. Experimental design

2.4.1. Study 1: Protection in young SPF White Leghorn chickens

To assess protection conferred by a single vaccination, day-old chicks were vaccinated with the rHVT-H5 vaccine (n = 10), or were sham-vaccinated and used as controls (n = 10) (Table 1). To assess protection conferred by prime-boost vaccination, day-old chicks (n = 40) were vaccinated with the rHVT-H5 vaccine and four weeks later (4 weeks of age) boosted with either rgH5N1 (n = 20) or RP-H5 (n = 20) vaccine. A sham control group (n = 20) received sterile, non-infected SPF allantoic fluid. Three weeks after boost (7 weeks old), the birds (n = 60) were challenged by the choanal route with estimated $10^{6.5}$ EID_{50} of Tk/MN/15 virus in a volume of 0.1 ml. The inoculum titer was subsequently verified as $10^{6.3–7.1}$ EID_{50} by back titration in embryonating chicken eggs.

2.4.2. Study 2: Protection in adult SPF White Leghorn hens for challenge 3 weeks post-vaccination

Adult 61-week-old hens were vaccinated with a single dose of either rgH5N1 (n = 20) or RP-H5 (n = 20) vaccine (Table 1). Sham-vaccinated control hens (n = 30) received sterile, non-infected SPF allantoic fluid. Three weeks after vaccination, all hens were challenged by the choanal route with estimated $10^{6.5}$ mean embryo infectious doses (EID_{50}) of Tk/MN/15 virus in a volume of 0.1 ml. The inoculum titer was subsequently verified as $10^{6.5}$ EID_{50} by back titration in embryonating chicken eggs.

2.4.3. Study 3: Protection in adult SPF White Leghorn hens for challenge 20 weeks post-vaccination

Adult 61-week-old hens were vaccinated with two doses of rgH5N1 (n = 20), 3 weeks apart (61 and 64 weeks old), or were sham-vaccinated and used as controls (n = 20) (Table 1). Twenty weeks after boost, all hens were challenged by the choanal route with estimated $10^{6.5}$ EID_{50} of Tk/MN/15 virus in a volume of 0.1 ml. The inoculum titer was subsequently verified as $10^{6.7}$ EID_{50} by back titration in embryonating chicken eggs. Mortality due to aging and other unspecified reasons reduced the group numbers to n = 14 (vaccinated group) and n = 17 (sham group) by challenge.

2.5. Sampling

All the birds were monitored daily for clinical signs and mortality. Severely sick birds were euthanized and counted as dead for the next day in mean death time (MDT) calculations. Oropharyngeal swabs (and in some groups also cloacal swabs) were collected at 2 and 4 days post-challenge (dpc) (also at 7 dpc in prime-boosted groups of Study 1) in 1.5 ml brain heart infusion media (Becton, Dickinson and Company, Sparks, MD) with penicillin (2000 units/ml; Sigma Aldrich), gentamicin (200 μg/ml; Sigma Aldrich) and amphotericin B (5 μg/ml; Sigma Aldrich). Up to 3 birds per group were necropsied upon death or euthanasia and tissues were collected in 10% buffered formalin (Thermo Fisher Scientific, Waltham, MA) for histopathologic evaluation. Serum samples were taken at time of vaccination, challenge (0 dpc), and termination (14 dpc). In Study 3, serum samples were also collected weekly between boost and termination. At 14 dpc, surviving birds were euthanized by cervical dislocation.

2.6. Serology

Hemagglutinin inhibition (HI) assays were carried out using H5 antigens specific for the vaccine seed viruses A/Gyrfalcon/Washington/40188-6/2014 (H5N8) (for rgH5N1 and RP-H5 vaccines) and A/Whooper Swan/Mongolia/3/2005 (H5N1) (WS/05) (surrogate homologous antigen for rHVT-H5 vaccine), and the challenge virus Tk/MN/15. The antigens were prepared as previously described [24] and the HI assays were performed according to standard procedures [25]. Titers were expressed as $\log_2$ geometric mean titers (GMT). Samples with titers below 3 $\log_2$ GMT were considered negative.

2.7. Determination of virus shedding from swabs

Total viral RNA from 250 μl of swab sample was added to Trizol and after the addition of chloroform the aqueous phase was used with the MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Inc., Austin, TX). The procedure for RNA isolation was carried out using the KingFisher magnetic particle processing system (Thermo Scientific, Waltham, MA) [26]. Quantitative real-time RT-PCR (RRT-PCR) was performed using primers and probe specific for type A avian influenza (AI) matrix gene [27]. Both reactions were carried out in a Smart Cycler II (Cepheid, Sunnyvale, CA) real-time PCR machine. The EID_{50s} of virus from the swab samples were extrapolated from the cycle thresholds by using standard curves generated from the known amounts of RNA of the challenge virus used [28]. Detection limits of each RRT-PCR run were calculated based on the R-square value of the standard curve, by setting the cycle threshold values equal to the number of cycles run. For statistical purposes, samples that were RRT-PCR-negative in this study were assigned a cycle threshold value of 1 cycle below the lowest detection point in the standard curve.

2.8. Statistical analysis

The D'Agostino and Pearson test was used to assess the normality of distribution of investigated parameters. All parameters in our study were not normally distributed. Mortality and number of birds shedding or seroconverting were tested for statistical significance with Fisher's exact test. Significant difference for mean viral titers in tissues between groups was analyzed using Kruskal-Wallis test or Mann-Whitney test (GraphPad Prism™ Version 5 software). A p-value of <0.05 was considered to be significant.

2.9. Histopathology and immunohistochemistry

Tissues in 10% formalin were processed for routine hematoxylin/eosin staining. Tissues were also processed for immunohistochemical staining using a mouse-derived monoclonal antibody (P13C11, developed at SEPRL) specific for type A influenza virus nucleoprotein, as previously described [29,30].

3. Results

3.1. Study 1: Protection in young SPF White Leghorn birds

Day-old birds were primed (rHVT-H5 vaccine) and challenged at four weeks of age, or prime-boosted (rHVT-H5 + rgH5N1 or rHVT-H5 + RP-H5 vaccines) and challenged three weeks after boost. After challenge, all the sham-vaccinated control birds showed acute severe clinical disease and death by 2 dpc, with a MDT of 2.0 dpc (Fig. 1). All the vaccinated birds remained clinically healthy for the duration of the challenge experiment (14 dpc) except for one bird single-immunized with rHVT-H5 vaccine that died on 8 dpc.

None of the sham-vaccinated control birds had detectable HI antibody titers before challenge (data not shown). In contrast, all the vaccinated birds had increasing detectable antibody titers against their corresponding vaccine seed strains pre-boost, pre-challenge, and at termination (Fig. 2a). Regarding antibody titers against challenge virus, only 8/20 rHVT-H5 + rgH5N1 vaccinated birds and 6/20 rHVT-H5 + RP-H5 vaccinated birds had seroconverted before the boost, with a mean titer of 3.1 and 3.5 $\log_2$ GMT, respectively (Fig. 2b). Prior to challenge, the highest antibody response against the challenge virus was observed in the rHVT-H5 + rgH5N1 group, with 8.0 $\log_2$ GMT (20/20 birds), followed by the rHVT-H5 + RP-H5 group, with 4.7 $\log_2$ GMT (18/20 birds), and finally the rHVT-H5 group, with 3.3 $\log_2$ GMT (8/10 birds). An anamnestic response at termination was observed for the rHVT-H5 and the rHVT-H5 + RP-H5 groups (Fig. 2b). The rHVT-H5 vaccinated bird that died had low antibody titers (3 $\log_2$ GMT) against WS/05 antigen and no detectable antibodies against Tk/MN/15 virus at challenge.

All the sham-vaccinated control birds (20/20) excreted high titers of virus in oropharynx (mean $10^{6.0}$ EID_{50}/ml) and cloaca (mean $10^{5.5}$ EID_{50}/ml) at 2 dpc (Fig. 3). Four of 10 rHVT-H5 vaccinated birds, 3/20 rHVT-H5 + rgH5N1 vaccinated birds, and 5/20 rHVT-H5 + RP-H5 vaccinated birds had low virus shed titers in oropharynx (mean $10^{1.7-1.9}$ EID_{50}/ml) at 2 dpc, and even fewer birds had lower cloacal titers. Mean oral shedding titers at 2 dpc of vaccinated birds were statistically lower than the sham-vaccinated controls ($P \leq 0.001$), as well as number of birds shedding. No significant differences between the three vaccinated groups were observed regarding shedding titers and number of birds shedding at 2 dpc, but significant differences were observed at 4 dpc; i.e. statistically more birds from the rHVT-H5 + RP-H5

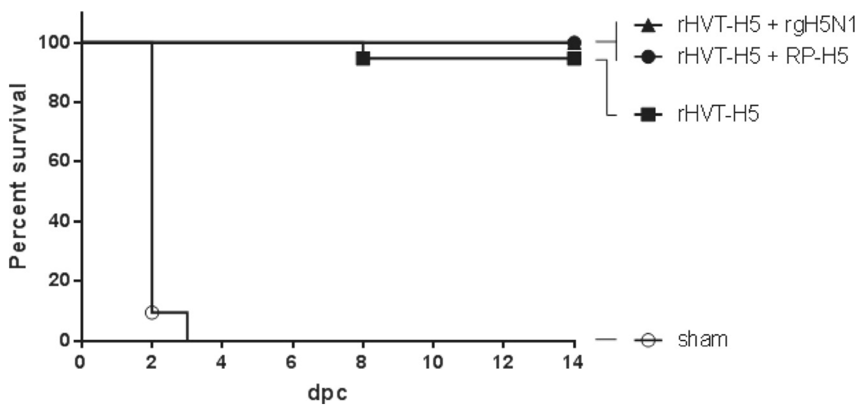

Fig. 1. Survival curve of Study 1.

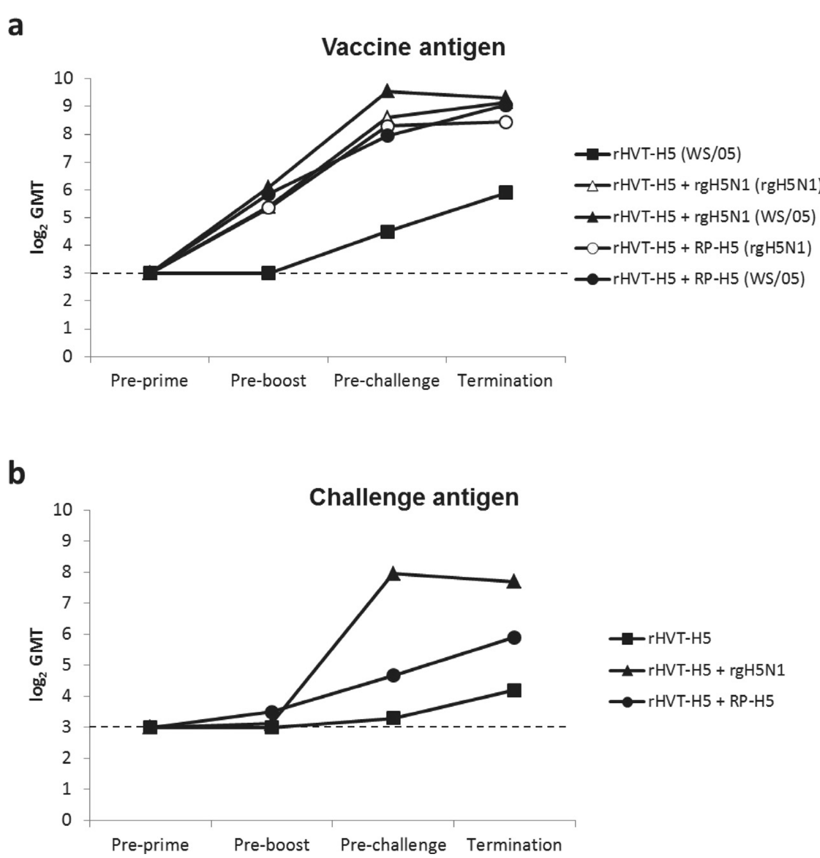

Fig. 2. Serology from vaccinated birds of Study 1. HI titers were assessed against (a) vaccine seed strain (in parenthesis) and (b) challenge strain Tk/MN/15. Titers are expressed as $\log_2$ GMT. Samples with titers below 3 $\log_2$ GMT were considered negative.

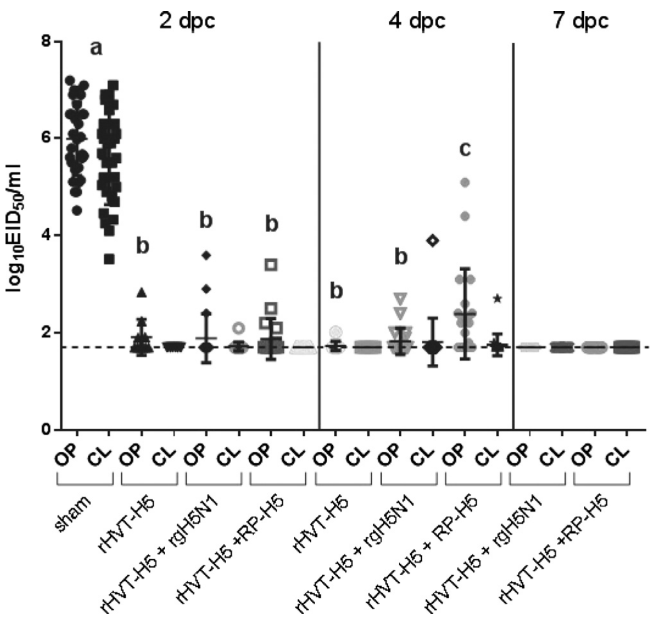

Fig. 3. Scatter plot of oral and cloacal shedding from vaccinated and sham birds of Study 1. Shedding titers are expressed as $\log_{10}$ with error bars included. The limit of detection of the qRRT-PCR was 1.8 $\log_{10}$ EID_{50}/ml; for statistical purposes, negative birds were given the value of 1.7 $\log_{10}$ EID_{50}/ml.

group excreted virus in oropharynx, with statistically higher titers, than both rHVT-H5 ($P \leq 0.004$) and rHVT-H5 + rgH5N1 ($P \leq 0.005$) vaccinated birds. By 7 dpc, none of the prime-boost vaccinated birds shed detectable virus in oropharynx and cloaca.

Three sham-vaccinated control birds were necropsied upon death at 2 dpc. Consistent gross lesions included necrotic comb and wattles, mottled spleen, necrotic and hemorrhagic pancreas, hemorrhagic digestive tract, hemorrhagic trachea, petechial hemorrhages on cloacal bursas, breast muscle, and pericardium, and renomegaly with parenchymal mottling. Multifocal necrosis with viral antigen was widespread in the parenchymal cells of most tissues, especially prominent in brain, heart, lung, spleen, pancreas, kidney, and adrenal gland. Viral antigen staining was frequently observed in capillary endothelial cells of various tissues but not as widespread as with H5N1 Gs/GD lineage viruses [29,31,32].

3.2. Study 2: Protection in adult SPF White Leghorn hens for challenge 3 weeks post-vaccination

Adult hens were vaccinated once with either rgH5N1 or RP-H5 vaccine and challenged three weeks later. After challenge, all the sham-vaccinated control birds showed acute severe clinical disease and death by 2 dpc, with a MDT of 2.1 dpc (Fig. 4). All the vaccinated birds remained clinically healthy for the duration of the challenge experiment (14 days) except for one RP-H5 vaccinated bird that was clinically ill by 2 dpc and died on 5 dpc.

None of the sham-vaccinated control birds had detectable HI antibody titers before challenge (data not shown). On the contrary, 20/20 of the rgH5N1 vaccinated birds had detectable antibody titers prior to challenge against the vaccine seed strain (mean 5.9 $\log_2$ GMT) and against the challenge strain (mean 6.5 $\log_2$ GMT). Of the RP-H5 vaccinated birds, 15/20 birds had detectable antibody titers prior to challenge against the vaccine seed strain (mean 5.6 $\log_2$ GMT), which was statistically lower than the number of birds seroconverting in the rgH5N1 group ($P = 0.047$); the RP-H5 vaccinated bird that died was among the birds that did not seroconvert. At termination, all the surviving vaccinated birds had detectable antibody titers against both the vaccine seed strain and the challenge virus, with 6.4 $\log_2$ GMT and 6.9 $\log_2$ GMT, respectively.

Sham-vaccinated control birds were shedding high titers of virus in oropharynx (30/30, mean $10^{6.4}$ EID_{50}/ml) and cloaca (15/20, mean $10^{7.0}$ EID_{50}/ml) at 2 dpc (Fig. 5). In contrast, only 9/20 rgH5N1 vaccinated birds had low virus shed titers in oropharynx (mean $10^{1.9}$ EID_{50}/ml) and 13/20 cloaca (mean $10^{3.4}$ EID_{50}/ml) at 2 dpc. Among the RP-H5 vaccinated birds, 16/20 birds were shedding intermediate titers in oropharynx at 2 dpc ($10^{3.1}$ EID_{50}/ml) and 4 dpc ($10^{3.5}$ EID_{50}/ml). Interestingly, virus titers shed in cloaca at 2 dpc were statistically higher than the corresponding titers shed in oropharynx in those groups tested for both shedding routes (i.e. sham birds and rgH5N1 vaccinated birds). Mean oral shedding titers at 2 dpc of all vaccinated birds, as well as number of birds shedding, were statistically lower than the sham-vaccinated controls ($P \leq 0.001$). When comparing oral shedding of both vaccinated groups at 2 dpc, rgH5N1 vaccinated birds shed significant lower titers by fewer birds than RP-H5 vaccinated birds ($P \leq 0.001$). Oral and cloacal virus titers shed by vaccinated birds at 4 dpc were statistically not different from corresponding titers at 2 dpc. The oral shedding in the RP-H5 vaccinated birds showed one sample with the highest virus titer, similar to the mean titer of the sham vaccinated birds, at 2 and 4 dpc (Fig. 5). Interestingly, this bird had no detectable antibody response and was the only vaccinated bird to succumb to the challenge virus, suggesting a total lack of development of protective immunity from the vaccine.

Three sham-vaccinated control birds were necropsied upon death at 2 dpc. Similar findings to the necropsied sham birds from Study 1 were observed, with additional grossly hemorrhagic ovaries and viral antigen staining in parenchymal cells of ovarian follicles.

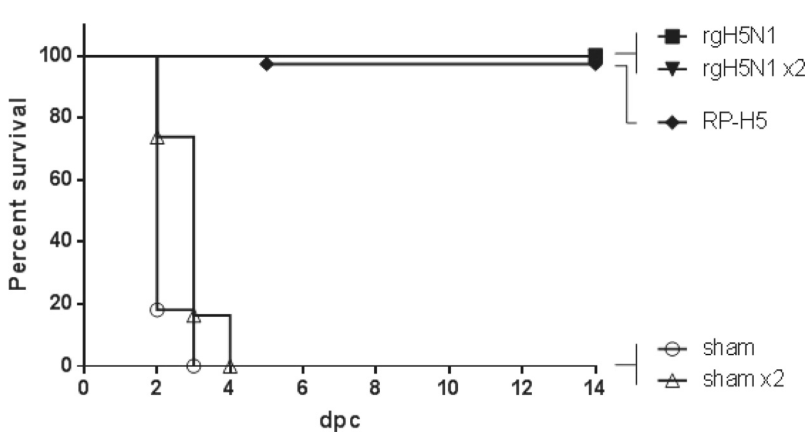

Fig. 4. Survival curve of Studies 2 and 3.

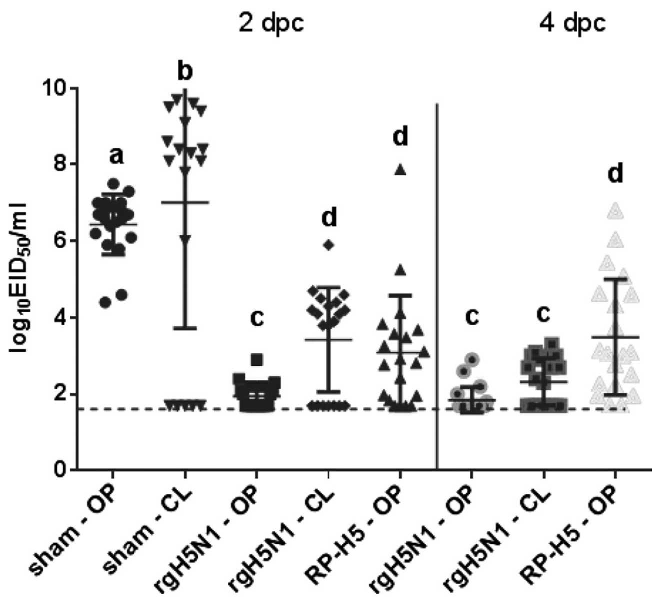

Fig. 5. Scatter plot of oral and cloacal shedding from vaccinated and sham birds of Study 2. Shedding titers are expressed as $\log_{10}$ with error bars included. The limit of detection of the qRRT-PCR was 1.8 $\log_{10}$ EID_{50}/ml; for statistical purposes, negative birds were given the value of 1.7 $\log_{10}$ EID_{50}/ml.

3.3. Study 3: Protection in adult SPF White Leghorn hens for challenge 20 weeks post-vaccination

Adult hens were vaccinated twice with rgH5N1 vaccine and challenged 20 weeks later. After challenge, all the sham-vaccinated control birds showed acute severe clinical disease and death by 3 dpc, with a MDT of 2.6 days (Fig. 4). All the vaccinated birds remained clinically healthy for the duration of the challenge experiment (14 days).

None of the sham-vaccinated control birds had detectable HI antibody titers before challenge (data not shown). In contrast, 100% (17/17) of the vaccinated birds had detectable antibody titers against the vaccine seed strain after the prime vaccination (7.0 $\log_2$ GMT), which increased and maintained until challenge (9.4 $\log_2$ GMT), and termination (10.6 $\log_2$ GMT) (Fig. 6). At termination, all the vaccinated birds also had detectable antibody titers against the challenge virus (8.0 $\log_2$ GMT, data not shown).

All the sham-vaccinated control birds (14/14) were shedding high titers of virus in oropharynx (mean $10^{7.7}$ EID_{50}/ml) at 2 dpc, while only 7/17 rgH5N1 vaccinated birds had low virus shed titers in oropharynx (mean $10^{2.7}$ EID_{50}/ml) at 2 dpc (Fig. 7). Mean oral shedding titers at 2 dpc of vaccinated birds were statistically lower than the sham-vaccinated controls ($P \leq 0.001$), as well as number of birds shedding. Oral virus titers shed by vaccinated birds at 4 dpc remained statistically not different from titers detected at 2 dpc.

4. Discussion

Our current control strategy for HPAI, a foreign animal disease, in the U.S. is to rapidly identify infected and exposed flocks and humanely cull the birds as quickly as possible to reduce the opportunity for the virus to transmit to other flocks. This approach has been effective in the past, but as the 2014–15 outbreak demonstrated, when the virus gets introduced onto large farms or areas of highly concentrated poultry production, the ability to quickly euthanize and dispose of infected birds can be overwhelmed, allowing virus to replicate to high titers with shedding of high

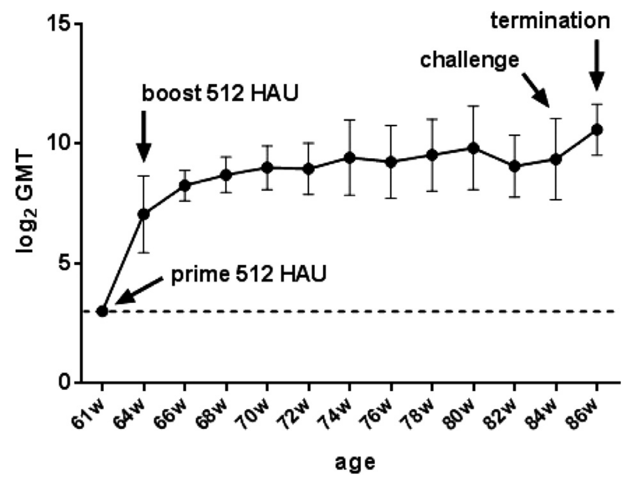

Fig. 6. Serology from vaccinated birds of Study 3. Follow-up HI titers against vaccine seed strain during 25 weeks (from prime vaccination to termination). Titers are expressed as $\log_2$ GMT. Samples with titers below 3 $\log_2$ GMT were considered negative.

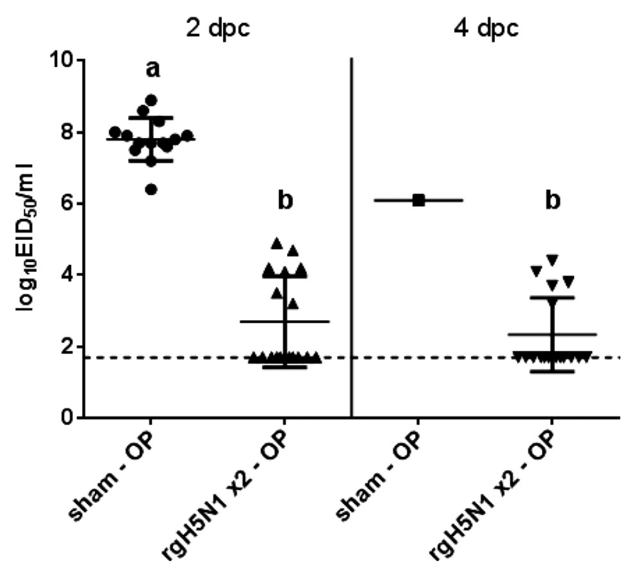

Fig. 7. Scatter plot of oral shedding from vaccinated and sham birds of Study 3. Shedding titers are expressed as $\log_{10}$ with error bars included. The limit of detection of the qRRT-PCR was 1.8 $\log_{10}$ EID_{50}/ml; for statistical purposes, negative birds were given the value of 1.7 $\log_{10}$ EID_{50}/ml.

quantities into the environment and spread. The principal alternative to a stamping-out approach is the use of vaccination in high risk areas to reduce the number of susceptible poultry and to reduce virus shedding if a vaccinated flock does become infected. Vaccination has been shown to be a viable alternative, but its use often results in export sanctions on poultry or poultry products because of fear that the commodities may contain HPAI virus, and importation of such products would introduce the disease [10]. If we overlook the trade implications for a moment, using vaccination in a country normally HPAI-free is difficult from both the regulatory and practical standpoints. The first issue is the availability of AI vaccines that are closely matched to the field strain so that clinical protection and reduction of virus shedding is achieved. From 2005 to 2010, the U.S. had a vaccine bank containing two inactivated H5 vaccines based on North American LPAI viruses and a recombinant fowl-pox virus vaccine with an HA insert from 1983 Ireland H5 virus (rFPV-H5). Single vaccination of chickens with the inactivated vaccines, the rFPV-H5, or the clade 2.2

rHVT-H5 did not produce adequate primary protection against challenge by an H5N2 and H5N8 clade 2.3.4.4 wild bird HPAI viruses, necessitating development of new vaccine seeds strains and inserts for recombinant vaccines [33].

Vaccine manufactures are unlikely to develop and license vaccines in a country that does not routinely vaccinate for a particular disease because of the lack of a defined market. In the U.S., the rFPV-H5 and rHVT-H5 AI vaccines had been licensed by the Center for Veterinary Biologics (CVB), but had never been used in the country. During the U.S. outbreak in 2014–2015, the Animal and Plant Health Inspection Service (APHIS) announced it would support enhancing the NVS by purchasing up to 500 million of doses of vaccine for possible use in future AI outbreaks [34]. This economic incentive helped spur the licensing of two additional vaccines, the rgH5N1 and RP-H5 vaccines, by CVB. Ultimately, three vaccines were purchased by the NVS for the emergency vaccine bank, although the exact circumstances for using the vaccine from the stockpile and the exit strategy to stop using vaccination have not been predetermined. The vaccine studies presented here, as well as other and future vaccine studies, are crucial to help guide the decision making process of whether vaccines should or should not be used. In this study, the protective efficacy of three NVS vaccines (rgH5N1, rHVT-H5, and RP-H5) was assessed by vaccinating and challenging layer chickens of different ages with clade 2.3.4.4 H5N2 HPAI virus. The vaccines tested in this experiment, based on their different expression of AI viral proteins, are compatible with existing DIVA strategies using serological and/or virological screening tests.

In Study 1, the protective efficacy of single (rHVT-H5) and prime-boost (rHVT-H5 + rgH5N1 or rHVT-H5 + RP-H5) vaccination regimes was assessed in White Leghorn chickens. All vaccine strategies conferred clinical protection (90–100% survivability) against clade 2.3.4.4 Tk/MN/15 challenge, which corresponded to high levels of protective immunity and significantly reduced virus shedding. Comparable results have already been described for single dose of rHVT-H5 against numerous different clades of HPAI H5N1 Gs/GD HPAI virus infection [35–42], confirming the broad cross-clade protection of the clade 2.2 insert in rHVT-H5 vaccine alone. Similar results were observed in single rHVT-H5 vaccinated turkeys challenged with homologous clade 2.2 virus [43]. The good clinical protection conferred by rHVT-H5 alone demonstrates that, although protection can be associated with the presence of HI titers when challenge and vaccine virus match genetically and antigenically, heterologous vaccines (e.g. 2.2 clade against 2.3.4.4 clade) can confer protection if they contain key epitopes for HA receptor binding and/or are efficient in inducing cell-mediated immunity [36,41,44].

Previously, a prime-boost immunization was identified as the best vaccination strategy for optimal results in the control of HPAI virus [45–47]. Nevertheless, in the present study similar levels of clinical protection and reduction of virus shedding were observed in single vs. twice vaccinated birds despite much high HI antibody titers in twice vaccinated poultry. Interestingly, the lower HI titers in the rHVT-H5 vaccinated birds did not negatively impact virus shedding reduction. However, the slightly weaker immune response mounted against the challenge virus by rHVT-H5 + RP-H5 vaccinated birds compared to rHVT-H5 + rgH5N1 vaccinated birds could explain the significantly higher virus shed from the former at 4 dpc. It is worth highlighting that protective immune response in the field is more difficult to achieve and can be hampered due to improper use of vaccines, interference by maternally derived antibodies, poor management, or co-infection with other pathogens. These factors may significantly reduce the success rate of vaccination programs under field conditions, necessitating booster vaccinations [45,48]. Therefore, the prime-boost immunization strategy would likely remain as the preferred choice [12].

Both the rgH5N1 and RP-H5 platform vaccines allow for rapid insertion of any influenza HA (or other) gene, making both attractive vaccine technologies due to constant antigenic shift and drift among influenza viruses [49,50]. In the face of an influenza outbreak, a quick turnaround is crucial to containment and eradication efforts. Regarding what type of vaccine would perform better as boost, a whole-virus inactivated vaccine (rgH5N1) and an RP-H5 vaccine were compared in Study 1, providing similar results. In Study 2, the protection efficacy of single vaccination with either rgH5N1 or RP-H5 vaccines was assessed in adult chickens challenged 3 weeks later; both of them provided complete clinical protection (95–100% survivability). Similar to Study 1, a single immunization with rgH5N1 vaccine seemed more efficacious than RP-H5 vaccine in reducing viral shed and providing a homogeneous serological response. The antibody response 3 weeks post-vaccination of the RP-H5 group appeared weaker in terms of the seroconversion rate. The reasons behind such heterogenic response could include biological individual variation within the experimental groups, lower immunogenicity of the vaccine at the dose and adjuvant used, individual variation in the response of an RNA (RP-H5) vaccine, as has also been reported in DNA vaccine studies in chickens [51], or poor efficiency of the delivery method [52]. Study 2 highlights that not only the genetic similarity between the vaccine and the virus challenge is relevant on protective efficacy, but other factors may also have an impact. Further research is needed to fully optimize the dose of RP-H5 vaccine to induce a higher immune response.

In Study 3, the long-term protection efficacy of double rgH5N1 vaccination was assessed in adult chickens challenged 20 weeks post-immunization. This study was designed because one of the most critical factors for vaccination efficacy in chickens with long production life is the number and timing of administration of vaccine doses. There are insufficient studies on the development and persistence of antibody levels in chickens in the field, and general trends are difficult to establish because of numerous variables, including differences in genetic lines of chickens, number of times the vaccine was administered, vaccine dose, and different adjuvants [48,53]. Boltz et al. (2009) were able to detect neutralizing antibodies 28 weeks after one dose of inactivated vaccine in chickens reared under field conditions, and 40 weeks after double vaccination [54]. Because of the cost, few studies have addressed the duration of vaccine-induced immunity experimentally, and they vary with vaccine formulation and overall health of the bird [48,53]. A single immunization with inactivated H5N1 vaccine protected SPF White Leghorns against lethal H5N1 HPAI virus experimental infection 12 weeks [55] and 138 weeks post-immunization [56]. In our study, vaccinated birds were protected from clinical signs and mortality 20 weeks post-boost, which corresponded to high levels of long-lasting protective immunity and significantly reduced virus shedding. It is known that protection can be correlated with HI serological titers when the challenge virus and vaccine virus are genetically and antigenically closely related [12]. If an adequate proportion of the flock has minimum HI antibody titers of 5 $\log_2$ GMT, birds are expected to be protected from mortality; titers of 7 $\log_2$ GMT are associated with reduction in virus replication and shedding [57,58]. In our study, antibody titers against the vaccine strain (clade 2.3.4.4, homologous to the challenge strain) prior to challenge were 9.4 $\log_2$ GMT. Considering such high titers, it is likely that antibody levels would have been maintained above the minimum titers for protection (5 $\log_2$ GMT) and replication (7 $\log_2$ GMT) for much longer than 20 weeks post-vaccination.

In conclusion, this study represents the scientific evidence supporting the efficacy of genetically matched vaccines for the control of clade 2.3.4.4 HPAI virus in young and adult White Leghorn chickens, should a similar outbreak occur and an emergency vaccination program was considered in the U.S. Practical aspects should be taken into account when selecting the vaccination program, such as the targeted species, the number of vaccinations, and the potential compliance of the DIVA principle. The data from these studies suggest a prime-boost strategy for optimal results in the field. Also, a vaccine with a quick turnaround should be favored for a rapid HPAI virus control.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

DES, DRK, and DLS designed the experiments. KB, CB, and DHL performed the experiments. KB processed the samples and analyzed the data. KB, CB, DHL, DLS, DRK, and DES wrote and reviewed the manuscript. All authors read and approved the final manuscript.

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